DNA Binding by 4-Methoxypyrrolic Natural Products. Preference for Intercalation at AT Sites by Tambjamine E and Prodigiosin

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The 4-methoxypyrrolic natural products contain a common 4-methoxy-2,2′-bipyrrole chromophore and exhibit promising anticancer, antimicrobial, and immunosuppressive activities. Herein, the ability of two representative members, tambjamine E (**1**) and prodigiosin (**2**), to bind calf thymus DNA (CT-DNA), polyd[G-C]$_2$, and polyd[A-T]$_2$ has been characterized using absorption and fluorescence spectroscopy. Scatchard plots showed that **1** occupies a site size (*n*) of ca. three base pairs and possesses affinity constants (*K*) ranging from 1 to 0.1 × 10^5 M^{-1}. Prodigiosin (**2**) binds DNA by mixed modes, as isobestic points were not evident in titration experiments. The neutral aldehyde precursor **4** was found to possess no measurable DNA binding affinity, indicating that the enamine structure of **1** and the pyrromethene of **2** are essential elements for DNA binding affinity. The enamine of **1** was found to undergo hydrolysis to **4** with a half-life ($t_{1/2}$) of 14.5 h at pH 7.4 and 37.5 °C. For the B-ring nitrogen atom of **1**, a pK_a value of 10.06 was also established. From fluorescence spectroscopy it was found that **1**, **2**, and **4** possess weak emission spectra in water that is increased in nonaqueous solvents. For **1** and **2**, DNA binding also increased the emission yield. Energy-transfer measurements suggested an intercalative binding mode, with preference for AT sites. The ability of distamycin to displace **1** and **2** from the helix also suggested that they intercalate from the minor-groove. This specificity differs from other unfused aromatic cations that bind by a minor-groove mode at AT sequences and intercalate at GC sites. Reasons for the specificity displayed by **1** and **2**, as well as the implications of our findings to their biological properties are discussed.

Introduction

The tambjamines (i.e., **1**),[1−4] prodigiosin (**2**),[6−14] and tetrapyrrole **3**[15] (Figure 1) belong to a family of 4-methoxypyrrolic natural products derived from bacterial and marine sources that exhibit promising antitumor,[5,7,10] antimicrobial,[5,10] and immunosuppressive activities.[16,17] Representative members shown in Figure 1 are derived from the aldehyde precursor **4**.[1,9,10,13,15] Condensation of **4** with the appropriate amine affords the enamine structure of the tambjamines, i.e., **1**.[1] Prodigiosin **2** is colored deeply red due to the planar pyrrolylpyrromethene chromophore and is cationic at neutral pH.[18] The tetrapyrrole **3** has been identified as the blue pigment found in tambjamine and prodigiosin fractions.[3,15]

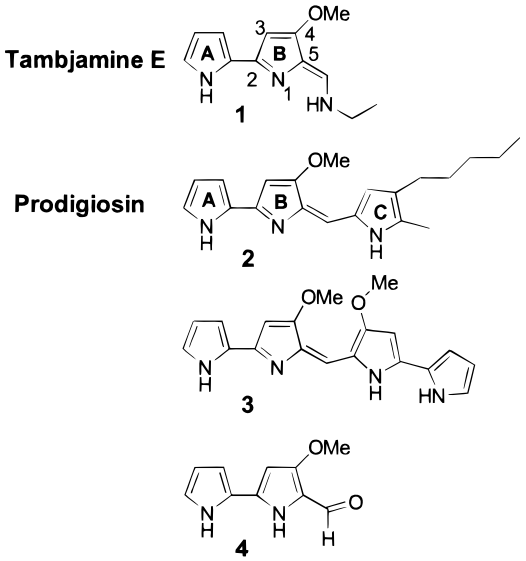

Figure 1. Structures of 4-methoxypyrrolic natural products **1**−**3** and aldehyde precursor **4**.

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Our interest in the 4-methoxypyrrolic natural products has been focused on gaining an understanding of their DNA-targeting properties. Previously, we demonstrated that tambjamine E (**1**, Figure 1) facilitates oxidative DNA cleavage in the presence of copper(II) without the aid of an external reductant.[19] This implied that Cu(I) was formed reductively through the concomitant oxidation of **1** to a π-radical cation. The tambjamine was found to bind DNA and Cu(II) effectively, and on the basis of quenching experiments that argued against participation of a freely diffusible hydroxyl radical, a copper−oxo species was proposed to initiate DNA strand scission.[19]

While our preliminary findings established DNA as a potential therapeutic target for **1**, information is lacking on the structural basis for DNA binding, specificity of copper-mediated DNA cleavage (if any), and nature of oxidative DNA damage (extent of H-abstraction from the deoxyribose sugars vs oxidation of DNA bases). Determination of these facets of tambjamine chemistry would facilitate an understanding of their mode of action in a biological system. Comparison to the corresponding DNA-targeting activities of prodigiosin **2**[20] and the tetrapyrrole **3** could then provide the necessary insight for the design and synthesis of superior polypyrrolic DNA targeting molecules that may serve as more effective antimicrobial or anticancer agents.

Here, we focus on the DNA-binding properties of tambjamine E (**1**) and prodigiosin (**2**) as representative members of the 4-methoxypyrrolic natural products. These compounds possess unfused aromatic ring systems that bear cationic charge at physiological pH. This latter property was found to be critical for DNA binding as the neutral aldehyde precursor **4** was found to lack the ability to bind DNA effectively. The fluorescent properties of **1** and **2** permitted us to gain insight into their mode of DNA binding. These compounds exhibit small emission intensities in water that is dramatically increased in organic solvents. This phenomenon was also observed for DNA binding. Energy transfer from the DNA helix (donor) to the 4-methoxypyrrolic natural product (acceptor) suggested that these compounds bind DNA by intercalation with a preference for AT sequences. This behavior differs from other unfused aromatic cations, such as DAPI,[21] furamidine,[22] and the bithiazole of bleomycin[23−26] (Figure 2), whose pharmacological properties have been correlated with their DNA targeting activities. For these compounds, a growing body of evidence implies that they bind AT sequences by a minor-groove binding mode, while intercalation is favored at GC sites.[21−26]

Our findings, together with our cleavage results,[19,20] show that the 4-methoxypyrrolic natural products represent an interesting class of DNA targeting agents. The bipyrrole coupled to the enamine or pyrromethene enable these compounds to bind DNA, coordinate redox-active metal ions such as Cu(II)[19] and facilitate oxidative DNA strand scission in the presence of Cu(II) without the aid of an external reducing agent.[19,20]

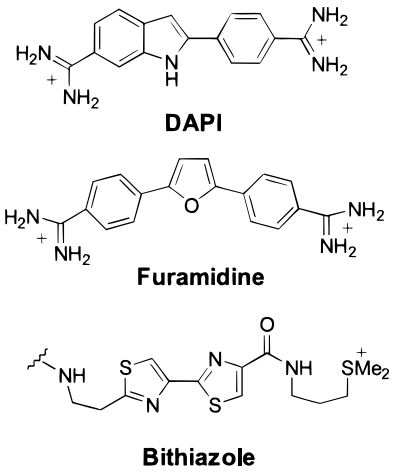

Figure 2. Structures of unfused aromatic cations that bind DNA.

Experimental Section

Compounds. The sample of tambjamine E (**1**) was obtained from the organic extracts of the marine ascidian *Atapozoa* sp., as described previously.[3,19] Concentrations of **1** were obtained from UV−vis measurements in methanol: (ϵ_{405} = 24,700 M^{-1} cm^{-1}).[3] Prodigiosin (**2**) was a gift from the Natural Products Division of the National Cancer Institute (NCI) and was received as a dark red powder. Confirmation of prodigiosin structure was obtained by ^{1}H NMR spectroscopy and electrospray mass spectrometry (ES^+): $[M + H]^+$ = 324.2. Stock solutions of **2** were prepared in dimethyl sulfoxide (DMSO), and concentrations were determined by UV−vis in 95% EtOH−HCl (ϵ_{535} = 112 000 M^{-1} cm^{-1}).[18] The bipyrrole aldehyde precursor **4** was derived from **1** through base hydrolysis using the protocol described by Faulkner.[1] The aldehyde **4** was obtained as a yellow residue, and its identity was verified by ^{1}H NMR,[1,5,10] and ES^+: $[M + H]^+$ = 191.1.[1] Concentrations of **4** were obtained from UV−vis (MeOH, ϵ_{364} = 6200 M^{-1} cm^{-1}).[1]

Buffers. Buffer solutions were prepared from the following: MOPS (3-(*N*-morpholino)propanesulfonic acid), MES (2-(*N*-morpholino)ethanesufonic acid, CHES (2-(cyclohexylamino)-ethanesulfonic acid), CAPS (3-(cyclohexylamino)propanesulfonic acid (Aldrich), using distilled, deionized water from a Milli-Q system. Sodium chloride or sodium perchlorate ($NaClO_4$) were added to adjust the ionic strength, and the pH was adjusted using NaOH.

DNA. Calf thymus DNA (CT-DNA, Sigma) was sonicated and phenol-extracted prior to use, while polyd[G-C]$_2$ and polyd[A-T]$_2$ (Sigma) were used without further purification. The polymer concentrations were determined by applying the appropriate molar extinction coefficients: ϵ_{260} = 12 824 M^{-1} cm^{-1} in base pair (bp) for CT-DNA, ϵ_{254} = 8400 M^{-1} cm^{-1} in base for polyd[G-C]$_2$,[27] and ϵ_{260} = 6800 M^{-1} cm^{-1} in base for polyd[A-T]$_2$.[28]

Kinetic Methods. Reactions were followed spectrophotometrically using a Hewlett-Packard (HP-8452) UV−vis spectrometer at 392 nm for **1** and 362 nm for **4**. Hydrolysis reactions were initiated by adding 2 μL of a stock solution of **1** (15 mM) in DMSO to a 3-mL cuvette containing 2 mL of

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buffer (10 mM) equilibrated at 25 or 37 °C. Mixing could be achieved within 2 or 3 s, and the data, which were analyzed using the ENZFITTER program, gave good first-order rate constants for at least 2 half-lives.

Proton Affinity. The pH measurements were obtained at 25 °C on a Fisher Scientific Accumet 910 pH meter using standard glass electrodes. Calibration was done using commercial buffers (BDH, pH 4.00, 7.00, and 10.00, all ± 0.01). For the pH range 8.5−11.0, the proton affinity of **1** was determined spectrophotometrically[29] at 25 °C using a 1-mL cuvette containing 1 mL of a 10 mM solution of CAPS, CHES, or NaOH, with 100 mM $NaClO_4$. To the sample cell was added 2 μL of a 15 mM stock solution of **1** in DMSO. UV−vis spectra were recorded by the overlay method in the wavelength range of 250−500 nm.

DNA Binding Affinity. Apparent equilibrium binding constants (K) for DNA binding by **1** were determined by UV−vis at 25 °C using a Hitachi U-2001 UV−vis spectrophotometer. Wavelength scans and absorbance measurements were made in 3-mL quartz cells of 1-cm path length containing 15 μM **1**, 50 mM buffer, and 10 or 100 mM NaCl. The DNA samples were typically added in 7.5 μM bp aliquots, and extinction coefficients of both the free and bound drug were determined at 392 nm, which represented the point of optimal spectral change.[19] For determination of the binding isotherms, absorbance values were converted into r (moles of **1** bound per mole of DNA bp) and free ligand concentrations (c). Values for the fraction of bound drug (α) between 0.2 and 0.8 were then used to determine K and n (the binding site size in DNA bp) based on the site exclusion model of McGhee and von Hippel.[30] The ENFITTER program was used to calculate nonlinear least-squares best-fit values for K and n.

Fluorescence Spectroscopy. Fluorescence spectra were obtained using a Hitachi F-2000 spectrometer at 25 °C. The following excitation (λ_{exc}) and emission (λ_{em}) maxima were utilized: **1**, λ_{exc} = 392 nm, λ_{em} = 450 nm; **2**, λ_{exc} = 520 nm, λ_{em} = 560 nm; **4**, λ_{exc} = 360 nm, λ_{em} = 425 nm. The fluorescence measurements involving DNA were carried out in 50 mM MES buffer pH 6.5 containing 10 mM NaCl. Energy transfer from the polynucleotide to the bound drug was measured from the excitation spectra of the complex in the wavelength range 220−310 nm. For **1**, excitation spectra were recorded at λ_{em} = 450 nm, while for **2** the spectra were acquired at 560 nm. The ratio of the fluorescence intensity of the bound versus free (I/I_o) was plotted against the excitation wavelength. Fluorescence quenching studies were carried out with the minor-groove binding agent distamycin (Sigma). Stock solutions of distamycin were prepared in DMSO and diluted to 3 mM in buffer ([distamycin] determined by UV−vis, ϵ_{305} = 34 000 M^{-1} cm^{-1}).[31] Solutions of **1** (15 μM) and **2** (4 μM) were prebound to 10 bp equiv of CT-DNA in 2.0 mL of buffer, and the distamycin solution was added in 3 μM aliquots.

Results

Hydrolytic Stability and Proton Affinity of Tambjamine E (1). To provide information on the aqueous stability of **1**, rate constants for the hydrolysis of the enamine were determined by UV−vis spectroscopy. The determinations were performed in the neutral to basic pH range (7.0−10.5) at 25 °C and at pH 7.4 at 37.5 °C. Figure 3 shows representative UV−vis data for the conversion of **1** to the aldehyde **4** in aqueous buffered media, pH 9.0, at 25 °C and ionic strength 0.1 M ($NaClO_4$). Monitoring the disappearance of the enamine (392 nm) or the appearance of **4** at 362 nm provided first-order rate constants in good agreement (±5%). All the values of k_{obs} (s^{-1}) are summarized in Table S1 (Supporting Information), which also contains rate constants for the hydrolysis of **1** at pH 7.4, 37.5 °C, under various ionic conditions. At 25 °C, rate constants for the hydrolysis (k_{obs}) increased from 0.857 × 10^{-5} to 47.7 × 10^{-5} s^{-1} as the pH was varied from 7.4 to 10.1, and at 37.5 °C the use of NaCl, $MgCl_2$, or $NaClO_4$ had little effect on k_{obs}. At the high pH region (9.5−10.5) it was also noted that **1** underwent a change in UV−vis characteristics showing a blue shift from λ_{max} = 392 nm to λ_{max} = 372 nm that was accompanied by a reduction in peak intensity (Figure S1, Supporting Information). Prodigiosin **2** also shows a similar pH-dependent change in absorbance (535 nm in acidic media, ca. 470 nm in alkali), and this has been attributed to deprotonation of the pyrromethene chromophore (pK_a = 8.25 in aqueous ethanol, 7.6 in aqueous dioxane[18]). The corresponding absorbance change for **1** (Figure S1) permitted determination of a pK_a value of 10.06 ± 0.03 (95% confidence interval). This result was attributed to deprotonation of the B-ring nitrogen atom.[5]

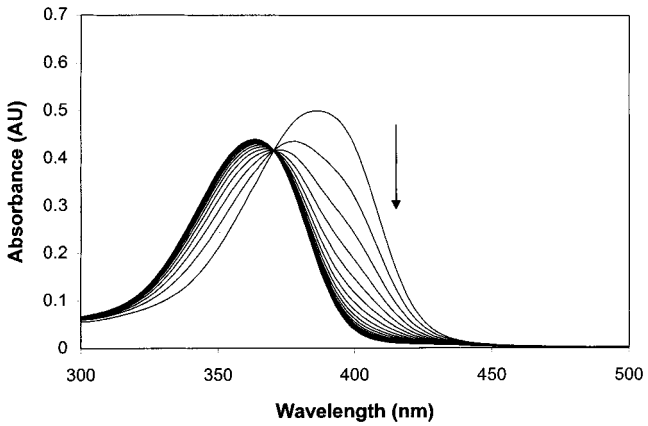

Figure 3. UV−vis spectroscopic data for the hydrolysis of **1** in 10 mM CHES buffer pH 9.0 containing 100 mM $NaClO_4$ at 25 °C. Arrow indicates loss of tambjamine absorption at 392 nm.

DNA Binding: Absorption Spectroscopy. The 4-methoxypyrrolic natural products have strong absorption bands in the visible regions that are well suited for DNA-binding studies. As described previously,[19] DNA binding by **1** leads to a reduction in the extinction coefficient at 392 nm that is accompanied by a shift to longer wavelengths. For **1**, the changes induced by binding to CT-DNA, polyd[A-T]$_2$, and polyd[G-C]$_2$ were similar. Hypochromicity typically fell in the 20−30% range, and bathochromic shifts were 2−9 nm. Quantitative analysis of the UV−vis data permitted determination of the intrinsic binding constant (K) and the binding site-size (n) in DNA bp, based on the site-exclusion model of McGhee and von Hippel.[30] Representative Scatchard plots are shown in Figure S2 (Supporting Information), while the data for DNA binding by **1** are summarized in Table 1.

The poor water solubility of prodigiosin **2** made the DNA-binding studies difficult. Solutions (4 μM) could be analyzed in aqueous media at pH 6.5, where the prodigiosin is predominately protonated.[18] However, even at 4 μM solutions of **2** precipitated from solution at pH 7.4 and above. At pH 6.5, isobestic points were not observed in DNA titration experiments. Figure 4 shows the changes in the UV−vis spectra of **2** upon additions of 10 bp equiv of CT-DNA (**A**), polyd[G-C]$_2$ (**B**), and polyd[A-T]$_2$ (**C**). At this concentration, the AT polymer caused

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Table 1. Summary of DNA Binding Data for Tambjamine E (1)[a]

DNA	[NaCl][b]	pH	% H_{392}[c]	B shift[d]	$K \times 10^{-4}$	n[e]
CT-DNA	10	6.5	15	3	8.6	3
CT-DNA	10	7.4	28	4	4.0	
CT-DNA	100	7.4	20	5	0.9	
CT-DNA	10	8.5	25	2	1.0	
poly d(GC)	10	6.5	24	6	7.3	2.6
poly d(GC)	10	7.4	32	7	3.1	
poly d(AT)	10	6.5	20	9	13.2	3
poly d(AT)	10	7.4	26	5	7.4	

[a] All experiments were conducted in 50 mM buffer (MES at pH 6.5, MOPS at pH 7.4, and CHES at pH 8.5) at 25 °C. [b] Concentrations of NaCl are in mM. [c] % hypochromicity (% H) refers to the reduction in the extinction coefficient of the absorbance of **1** at 392 nm at saturation. [d] Bathochromic (red) shift measured in nm. [e] Binding constants were determined from the McGhee−von Hippel equation, where *K* is the apparent binding constant (M^{-1}) and *n* is the site size in DNA base pair (see Figure S2 in the Supporting Information). The estimated error in the *K* values was ±5%.

a bathochromic shift of 20 nm (spectrum **C**), while shifts of only 6 nm were observed for CT-DNA (spectrum **A**) and polyd[G-C]$_2$ (spectrum **B**). Near saturation (**2**/DNA = 1:20 bp), red specks formed in the aqueous media, suggesting that the prodigiosin **2**/DNA complex precipitated from solution. Attempts to circumvent this problem by adding 20 vol % DMSO, dioxane, or triethylene glycol failed to prevent precipitation. These factors prohibited accurate determination of equilibrium binding constants.

In contrast to **1** and **2**, the aldehyde precursor **4** showed no changes in its absorption at 362 nm upon addition of DNA (data not shown). As described in further detail below, these results were consistent with the inability of **4** to bind DNA effectively.

Fluorescence Spectroscopy. In water, **1**, **2**, and **4** were found to exhibit weak emission spectra. Thus, we sought to study their emission profiles in various media, and Table 2 lists the relative emission intensities (compared to that observed in ambiently oxygenated water) for **1**, **2**, and **4** at a given concentration. Across the series, the data shows that in going from H_2O to MeOH to $CHCl_3$, the emission intensities increase, and this was especially the case for the bipyrrole aldehyde **4**. In MeOH, increased concentrations of **2** quenched the fluorescence spectra and caused a shift to longer wavelengths (Figure 5). The result in Figure 5 was attributed to aggregation of the prodigiosin, as other cationic pigments, such as ethidium[32] and the cyanine dyes,[33−35] are known to form aggregates in solution that exhibit quenched fluorescence compared to their monomeric counterparts.[32−35] In $CHCl_3$, the effect of a proton donor (CH_3COOH) on the emission yields of **1**, **2**, and **4** was also examined. These experiments were carried out to determine whether quenching due to excited-state proton transfer[36−38] plays a role in the weak emission yields of these compounds in water. However, in this case, additions of CH_3COOH had virtually no effect on the emission spectra of **1**, **2**, and **4** (data not shown).

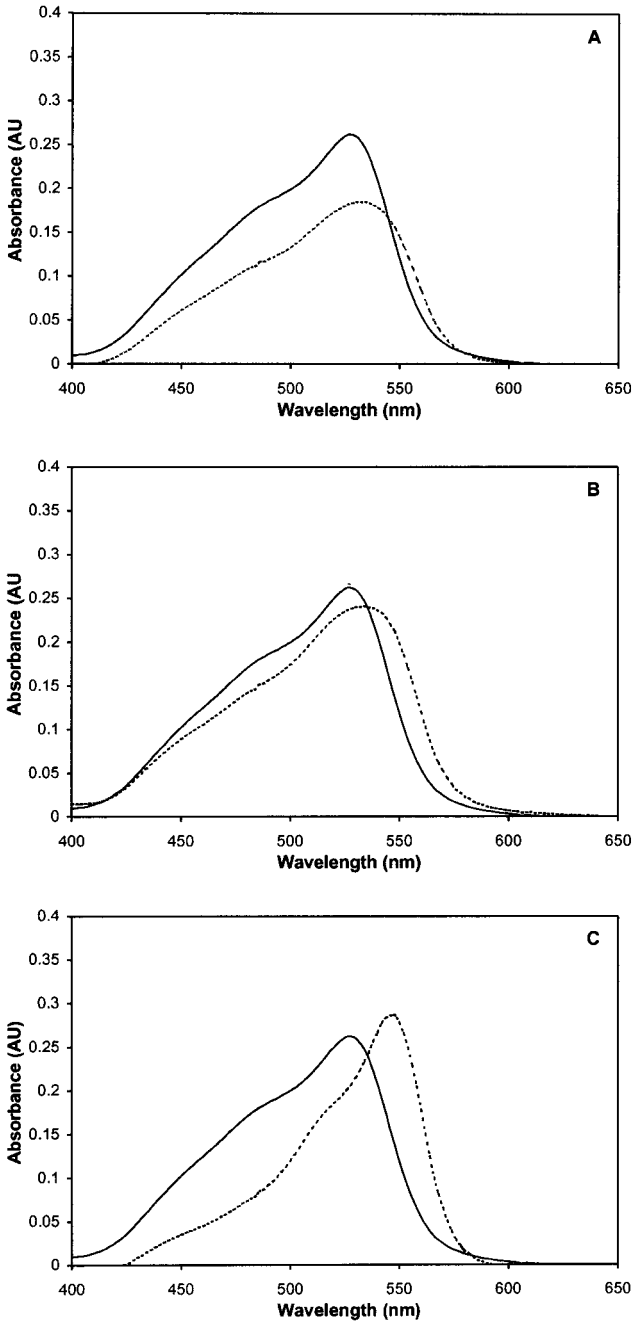

Figure 4. UV−vis spectra of prodigiosin **2** (4 μM) in the absence (solid line) and presence (dotted line) of 10 bp equiv of CT-DNA (**A**), polyd[G-C]$_2$ (**B**), and polyd[A-T]$_2$ (**C**) in 50 mM MES buffer pH 6.5 containing 10 mM NaCl.

Table 2. Fluorescent Data for 1, 2, and 4 in Different Media[a]

medium	**1** (15 μM)	**2** (4 μM)	**4** (5 μM)
H_2O	1.0	1.0	1.0
MeOH	1.6	1.4	9.0
$CHCl_3$	2.4	2.6	18.3

[a] Values are given as relative emission intensities in the indicated medium compared to that observed in ambiently oxygenated water alone under the same experimental conditions. The estimated error in the relative emission intensity values was ±0.1.

We then utilized fluorescence spectroscopy to study DNA binding by **1**, **2,** and **4**. Addition of excess DNA (10

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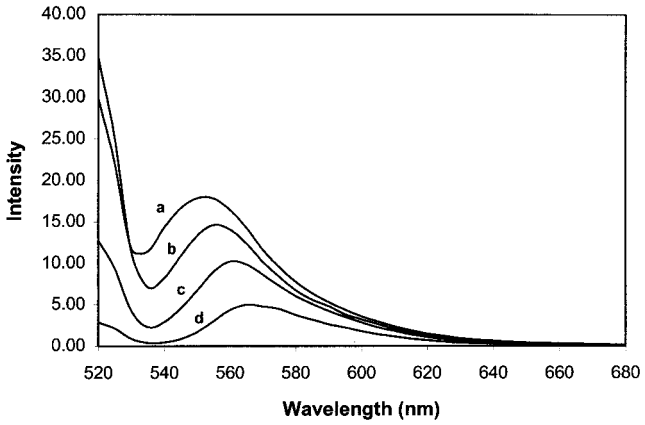

Figure 5. Concentration dependence on the emission spectrum of **2** in MeOH. Spectrum: (a) 4 μM; (b) 8 μM; (c) 16 μM; (d) 64 μM.

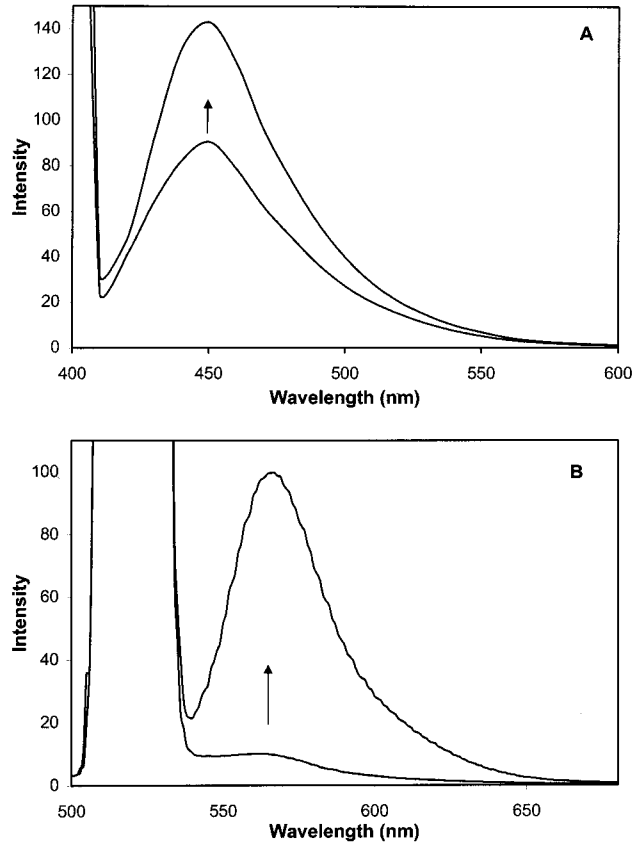

Figure 6. Emission spectra of **1** (15 μM, A) and **2** (4 μM, B) in the absence and presence of 10 bp equiv of CT-DNA, in 50 mM MES buffer pH 6.5 containing 10 mM NaCl. Arrow indicates enhancement of emission upon addition of DNA.

bp equiv) led to an increase in the intensity of emission for **1** and **2**, but not for **4**, consistent with its inability to efficiently bind DNA. For **1** and **2**, the intensity of emission was also sensitive to the nature of the DNA substrate, with the greatest intensity being noted for addition of the AT polymer. Figure 6 shows the changes in emission intensity for tambjamine E **1** (Figure 6A) and prodigiosin **2** (Figure 6B) upon binding CT-DNA. The relative emission intensities for binding to the various DNA substrates are summarized in Table 3.

Insight into the mode of DNA binding by **1** and **2** in the presence of 10 bp equiv of DNA was provided by

Table 3. Fluorescent Data for DNA Binding by 1 and 2[a]

DNA	**1** (15 μM)	**2** (4 μM)
none	90.6 (1.0)[b]	9.8 (1.0)
CT-DNA	143.0 (1.58)	142.0 (14.5)
poly d(GC)	167.5 (1.85)	99.7 (10.2)
poly d(AT)	172.7 (1.91)	271.0 (27.7)

[a] All experiments were conducted in 50 mM MES buffer at pH 6.5 containing 10 mM NaCl. DNA was present in 10 M base pair equivalents (150 μM for **1**, and 40 μM for **2**). [b] Values refer to emission intensities using λ_{exc} = 392 nm for **1** and λ_{exc} = 520 nm for **2**. Values in parentheses are relative emissions compared to **1** and **2** in the absence of DNA. The estimated error in the relative emission intensity values was ±0.1.

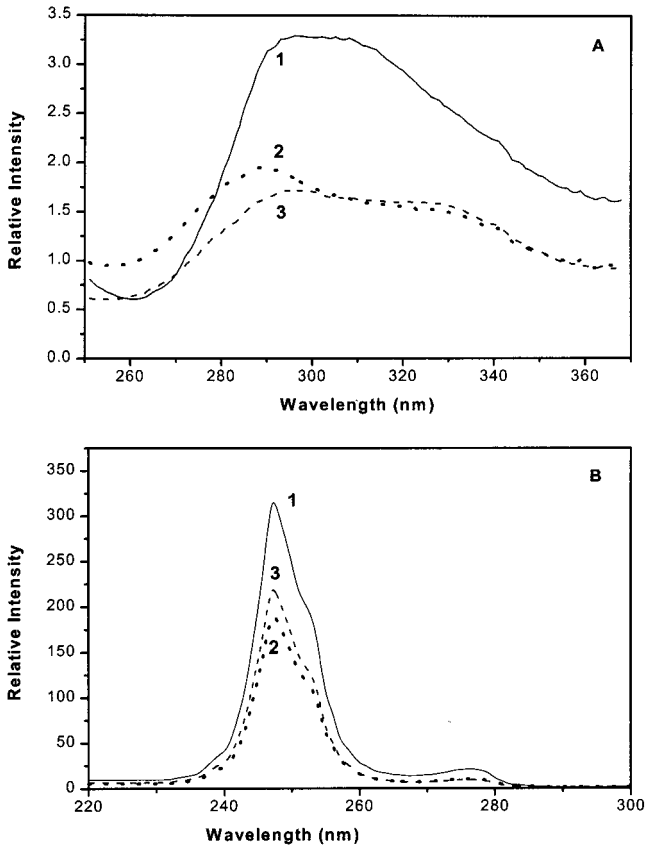

Figure 7. Ratio of the fluorescence intensity of **1** (15 μM, A) and **2** (4 μM, B) in the absence and presence of 10 bp equiv of (**1**) poly$d[A\text{-}T]_2$, solid line, (**2**) CT-DNA, dotted line, and (**3**) poly$d[G\text{-}C]_2$, dashed line, in 50 mM MES buffer pH 6.5 containing 10 mM NaCl.

fluorescence energy-transfer measurements. Le Pecq and Paoletti[39] have shown that the energy of UV absorbance by DNA (donor) base pairs may be efficiently transferred to an intercalated fluorophore (acceptor). Groove binders do not show energy transfer, and thus, these measurements have proven to be a valuable tool to establish an intercalative binding mode.[40,41] Figure 7 shows the results from the energy transfer measurements. For tambjamine E **1** (Figure 7A), a clear peak at ca. 290 nm, at the red edge of the DNA absorption spectrum, was observed. The intensity was the largest for the AT polymer, and the efficiency of sensitization decreased with increasing GC content. Control experiments where changes in the emission of **1** at 450 nm were monitored also showed a

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(41) Suh, D.; Chaires, J. B. *Bioorg. Med. Chem.* **1995**, *3*, 723.

clear increase in intensity with excitation at 290 nm, but not at 310 nm, where the DNA has no absorbance.

Sensitization of the prodigiosin fluorescence by the DNA bases was very efficient, giving rise to a peak centered at ca. 250 nm (Figure 7**B**). In this case, all three DNA substrates transferred energy to the prodigiosin **2**, but again transfer from the AT polymer was the most efficient.

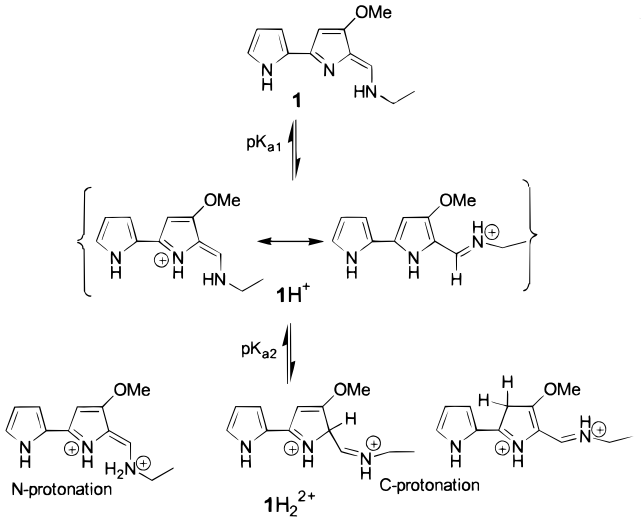
Additional information was obtained by examining the ability of the minor-groove binding agent distamycin[31,42] to displace **1** and **2** from the helix. These experiments could be easily carried out using fluorescence spectroscopy, as displacement should quench the emission of DNA bound **1** and **2**. Figure S3 (Supporting Information) shows that additions of distamycin quenched the emission intensity of **1** and **2** (both in the presence of 10 bp equiv of CT-DNA). Control experiments in MeOH showed that additions of distamycin had little effect on the emission intensities of **1** and **2**. These results suggested that in H_2O distamycin displaced **1** and **2** from the helix, implying that **1** and **2** intercalate from the minor-groove.

Discussion

Hydrolytic Stability and Proton Affinity of 1. The tambjamines are members of the 4-methoxypyrrolic natural products that contain an enamine structure. Many of the tambjamines have been isolated from marine organisms, such as sea squirts, that use them as chemical defenses against predators.[1−4] Derivatives have also been obtained from bacteria, and here, a compound designated as BE-18591 was shown to exhibit antitumor and antimicrobial activities.[5] In these studies, it was also noted that the aldehyde **4** was isolated under neutral conditions from the mycelium of the strain *Streptomyces* sp. BA18591.[5] Since **4** is the hydrolysis product of many tambjamines,[1] we sought to characterize its rate of formation from **1** under a range of conditions including those at physiological pH and temperature. During the course of these studies, we were also able to establish a pK_a value (10.06 ± 0.03) for deprotonation of **1** as the neutral species (λ_{max} = 372 nm in 10 mM NaOH) could be distinguished from the protonated species **1**H$^+$ (λ_{max} = 392 nm at pH 8.5). The 20 nm hypsochromic shift of the absorbance for **1** in base was consistent with deprotonation of the B-ring N atom.[5,18,43]

To account for the hydrolytic behavior of **1**, it is informative to consider its possible protonated forms. Thus, as shown in Scheme 1, protonation of neutral **1** (pK_{a1}) would yield the monocationic species **1**H$^+$ (resonance structure involving the exocyclic N is also shown). The geometry about the double bond for **1**H$^+$ where the positive charge is located on the B-ring N atom is shown to have the *Z* configuration.[5] The resonance form where the positive charge is located on the exocyclic N atom is shown to possess the *E* configuration, where it may be possible to form a hydrogen bond with the exocyclic oxygen of the B ring. For the tambjamine derivative BE-18591, the *Z* configuration for **1**H$^+$ was inferred from NMR studies in HCl−$CDCl_3$.[5] The hydrochloride salt of

Scheme 1. Proposed Pathways for Protonation of Tambjamine E (1)



BE-18591 was found to possess an exchangeable proton at 13.7 ppm, which was ascribed to the B-ring N atom based on its long-range $^{13}C-^1H$ couplings with C2 and C5 (Figure 1).[5]

Since the tambjamines contain an enamine structure, further protonation of **1**H$^+$ to yield **1**H$_2^{2+}$ (pK_{a2}) could occur at the exocyclic nitrogen of the enamine (N protonation) or at C-3 or C-5 (C-protonation).[44] While we did not establish a value for pK_{a2} or observe further UV−vis changes in **1**H$^+$ in the pH range 4−8.5, on the basis of pK_a values for a range of enamines,[44] pK_{a2} is expected to be significantly lower than pK_{a1} (10.06). For example, simple enamines have pK_a values for the N atoms that are typically 2−4 pH units below the parent amine (ethylamine, pK_a = 10.63[45]).[44] Coward and Bruice[46] have also reported on the nitrogen basicities of some β-cyano enamines (where the lone-pair electrons on the N atom can be delocalized into the cyano group), and these have values ranging from −2.3 to +3.5. Stabilization of the enamine base through resonance and destabilization of the N-protonated conjugate acid by the polar electron-withdrawing effect of the alkene provide a rationale for the decrease in N basicity.[44]

The pK_a value for **1**H$^+$ (10.06) is ca. 2−3 pH units above the corresponding pK_a of prodigiosin **2** (Figure 1).[18] This increase in pK_a for **1**H$^+$ seems consistent with the ability of the lone-pair electrons on the exocyclic N atom to stabilize the protonated species **1**H$^+$ more effectively through resonance than the pyrrole N-atom lone-pair electrons of **2**. However, as described by Rizzo and coworkers for a synthetic derivative of **2**[47] the proton affinity of the prodigiosins is more complicated than originally anticipated.[18] Here, they have been able to distinguish two geometrical isomers (α and β forms) that have very different pK_a values, i.e., pK_α = 8.23, pK_β = 5.4 (Figure 8). The pK_a for the α rotamer is influenced

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(43) The B ring of tambjamine E (**1**) may be compared to an azafulvene that is known to show a bathochromic shift of ca. 20 nm when solutions are acidified. Sammes, M. P. In *Pyrroles. Part One: The synthesis and the physical and chemical aspects of the pyrrole ring*; Jones, R. A., Ed.; John Wiley & Sons: New York, 1990; Chapter 4.

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(47) Rizzo, V.; Morelli, A.; Pinciroli, V.; Sciangula, D.; D'Alessio, R. *J. Pharm. Sci.* **1999**, *88*, 73.

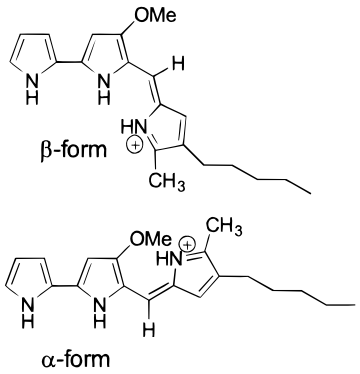

Figure 8. Structures of the α- and *β*-conformers of prodigiosin **2**. Taken from ref 47.

by hydrogen bonding between the protonated nitrogen of the C ring and the exocyclic oxygen of the B ring, as found in the crystal structure of a prodigiosin analog.[12] Thus, in analogy to **2**, the pK_a determined for **1**H$^+$ may involve contributions from both the *Z* configuration and the *E* configuration where hydrogen bonding to the exocyclic oxygen atom may influence the pK_a (Scheme 1).

The hydrolysis data for **1** (Table S1, Supporting Information) show that the enamine has a half-life of $t_{1/2}$ = 14.5 h at pH 7.4, 37.5 °C, containing 100 mM NaCl. The rate of hydrolysis was found to be slightly faster in the absence of salt ($t_{1/2}$ = 12.2 h), and the presence of a divalent metal ion such as Mg^{2+}, or change of the counterion to ClO_4^-, did not dramatically affect the rate. Stabilization of the cationic ground state (i.e., **1**H$^+$, Scheme 1) by salt, as opposed to the neutral product **4**, may provide a rationale for the slower rate of hydrolysis in the presence of 100 mM salt. At 25 °C, the effect of pH on the rate of hydrolysis was also examined (Table S1). As noted for other enamines,[46,48,49] the rate of hydrolysis was influenced by alkaline, and generally, an increase in rate was found with increased pH.

DNA Binding. The ability of the 4-methoxypyrrolic natural products to bind DNA was initially investigated using UV−vis spectroscopy. For tambjamine E (**1**), DNA binding was accompanied by small bathochromic shifts (2−9 nm, B shift, Table 1) and decreases in peak intensity (hypochromicity, % H 20−30%, Table 1) of the absorbance at 392 nm. These changes permitted determination of intrinsic binding constants (*K*) and binding site sizes (*n*) for DNA binding by **1**, where *K* values ranging from 1 to 0.1×10^5 M^{-1} and site sizes of ca. 2.5−3 base pairs were found. Binding constants were diminished by increased salt concentration (NaCl, 10 mM vs 100 mM) and increased pH (6.5 vs 8.5). These results were consistent with the importance of the cationic charge for **1**, and in support of this hypothesis was the finding that the neutral aldehyde **4** lacks the ability to bind DNA effectively. The *K* values ($1.0-0.1 \times 10^5$ M^{-1}) were also typical for unfused aromatic cations,[21] including the free bithiazole[50,51] of bleomycin (Figure 2), that bind DNA by (partial) intercalation.[22,52,53] For furamidine (Figure 2) and other unfused aromatic cations that bind AT sites by a minor-groove mode, much higher binding constants (ca. 1×10^7 M^{-1}) have been determined.[22]

For prodigiosin **2**, addition of 10 molar bp equiv of CT-DNA caused 30% hypochromicity and a 6 nm bathochromic shift (Figure 4A). Much smaller hypochromicity was noted for binding to polyd[G-C]$_2$ (Figure 4B), while binding to ployd[A-T]$_2$ caused a 20 nm bathochromic shift (Figure 4C). Binding to DNA was also accompanied by precipitation as **2** became fully bound to the DNA substrate. The same effect was noted in solutions containing 20 vol % DMSO, CH_3CN, dioxane, or triethylene glycol. These results were surprising since we initially anticipated that DNA binding would facilitate water solubility. However, in view of Rizzo's results,[47] it is interesting to speculate that **2** may display a conformational preference in its binding to DNA. Thus, as shown in Figure 8, if the *β* form of **2** (pK_a = 5.4) is the preferred binding conformation, then the pK_a of the bound prodigiosin would drop below the pH of the solution (6.5), which may cause precipitation as noted for free **2** in solutions at pH 7.4 and above.

Much more insight into the nature of DNA binding by **1** and **2** was obtained by fluorescence spectroscopy where addition of DNA was accompanied by an increase in emission intensity (Figure 6, Table 3). Fluorescence enhancements observed for molecules upon DNA binding,[38,39] or binding to other substrates such as cyclodextrins,[54] have been ascribed to decreases in degrees of freedom of molecular motion, prevention of collisional deactivation, restricted conformations, favorable microenvironmental polarity effects, and shielding of the excited state from water molecules or other species present in the bulk aqueous solution, among others.[54] For **1** and **2**, a combination of these factors is expected to play a role. For example, that the emission yield of **1** and **2** was increased as the polarity of the solvent decreased (Table 2) suggested that the emission enhancements for DNA binding likely involved a reduction in their exposure to water. Presently, we attribute this finding to the fact that **1** and **2** possess NH groups that can be efficiently quenched by water molecules due to formation of exciplexes from the interaction of water with the excited state of the fluorophore.[55] Prevention of collisional deactivation or self-quenching through aggregation is also expected to play a role, especially for prodigiosin **2**, as increased concentrations of **2** in MeOH led to quenched fluorescence (Figure 5) that was consistent with aggregation.[33−35]

Based on the premise that the increased yields of emission represent, in part, a reduction in exposure to water, it was possible to assess how the solvent accessibility for binding by **1** and **2** varies as a function of DNA substrate. As noted by Barton,[56] solvent accessibility is important to establish since molecules that exhibit little solvent accessibility are less likely to interact with exogenous species in solution, such as diffusible metal ions for activation of oxidative DNA cleavage.[19,20] The

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data in Table 3 suggested that the AT polymer provided the greatest protection from water, as its addition caused the greatest increase in emission intensity. The data also show that changes in emission were much more dramatic for DNA binding by **2**, which may imply that the NH groups of **2** are more shielded from solvent and are placed in the interior of the helix.

Evidence for an intercalative binding mode was obtained from energy-transfer measurements. For **1**, only the AT polymer acted as an efficient donor, and the efficiency of sensitization decreased with increasing GC content (Figure 7A). Since energy transfer can only occur if the bound drug is in close contact with, and oriented parallel to, the DNA base pairs, these data suggested that **1** binds by an intercalative mechanism with a preference for AT sites.[39−41] Intercalation would also be expected to provide greater shielding from the aqueous solvent than an outside binding mode, and this was consistent with the emission data for **1** where the AT polymer caused the greatest increase in emission intensity (Table 3).

For prodigiosin **2**, energy transfer from the DNA was very efficient and showed an intense band at 250 nm for all three DNA substrates (Figure 7B). While we currently lack high-resolution data on DNA binding by **1** and **2**, it is interesting to speculate that the intense band at 250 nm represents energy transfer to the C ring of **2**. The C-pyrrole ring of **2** is lipophilic due to the pentyl chain, and as discussed by Hecht and co-workers,[58,59] lipophilic groups bind DNA through interaction with the least polar component of the duplex, i.e., with the interior.

Rationale for Intercalation at AT Sequences. For the 4-methoxypyrrolic natural products, contributions from hydrogen-bonding interactions, electrostatics, and van der Waals contacts are expected to play a role in DNA binding. While the structural origin for the intercalation preference at AT sites by **1**, and partly by **2**, at this point is unclear, certain aspects of the interaction of **1** and **2** with DNA can be uncovered through consideration of the Watson−Crick base pairs (Figure 9), which offer different binding possibilities. That the minor-groove binding agent distamycin displaces **1** and **2** from the helix (Figure S3, Supporting Information) suggests strongly that they intercalate from the minor-groove of DNA. If we consider electrostatic interactions, theoretical calculations indicate that AT sites have a high negative potential in the minor-groove, while GC sites have a positive potential due to the guanine amino group.[60] Thus, intercalation may be favored at AT sites due to unfavorable electrostatic interactions between the positively charged aromatic system of **1** and **2** with the guanine amino group at GC sites. In terms of hydrogen-bonding interactions, it can also be argued that hydrogen bonding from the pyrrole NH atoms (donor) to the N atoms and O atoms of the AT base pairs (acceptors) are more favorable than the corresponding interactions with the GC sites. These factors could contribute to the preference for intercalation at AT sites by **1** and **2**.

The DNA binding specificity displayed by **1** and **2** differs from other unfused aromatic cations, such as

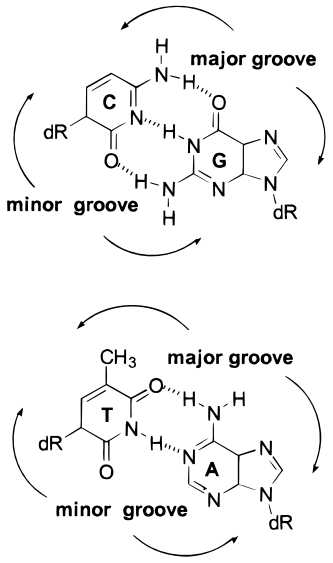

Figure 9. Structures of the Watson−Crick DNA base pairs.

DAPI, furamidine, and the bithiazole of bleomycin (Figure 2). For DAPI[21] and furamidine,[22] Wilson and co-workers have shown that they bind AT sites by a minor-groove mode, while intercalation is favored at GC sequences. For the minor-groove mode, the phenylamidine group is critical, as hydrogen bonding from this group to the minor-groove at AT base pairs anchors the compounds in the minor-groove,[22] while the neutral aromatic system interacts with the base pairs by van der Waals contacts. At GC sites, it is more favorable to bind by intercalation where the neutral aromatic system can interact with the base pairs through π-stacking interactions and unfavorable electrostatic interactions with the exocyclic amino group of guanine are avoided. The bithiazole of bleomycin may also be grouped with the unfused aromatic DNA binding agents. For the free bithiazole, a (partial) intercalation binding mode has been advanced.[52,53] However, when the bithiazole is part of the entire bleomycin molecule, both (partial) intercalation[24,26] and minor-groove binding models[23,25] have been reported, and not surprisingly, the minor-groove model has the bithiazole in the minor-groove of AT base pairs.[23,25] For **1** and **2**, the cationic charge is part of a planar aromatic system, and this aspect distinguishes them from the infused aromatic cations presented in Figure 2 and provides a rationale for their different specificity.

Conclusions

The 4-methoxypyrrolic natural products that include the tambjamines (i.e., **1**) and prodigiosin **2** have been known for some time to possess anticancer and antimicrobial activities. While the therapeutic target for these activities has not been established, we speculated that they would target DNA effectively based on their structural similarities to other unfused aromatic cations (Figure 2) that are known for their ability to bind DNA. In support of this hypothesis was our recent studies demonstrating the ability of **1** to facilitate oxidative DNA strand scission in the presence of Cu(II).[19] In the present paper, we have presented evidence that tambjamine E (**1**) and prodigiosin **2** bind DNA by intercalation with a preference for AT sites. The neutral aldehyde precursor **4** was found to lack the ability to bind DNA effectively,

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indicating that the enamine of **1** and pyrromethene of **2** are essential elements for DNA binding affinity. With regard to the therapeutic potential of these compounds, the tambjamines with the enamine structure are prone to hydrolysis, which may render them inactive since the hydrolysis product, i.e., **4**, lacks the ability to bind DNA effectively. Prodigiosin **2** is more stable, but possesses poor water solubility, which limits its use as a DNA targeting agent. However, attachment of water-soluble cationic groups to the prodigiosin carbon skeleton could result in very effective DNA targeting molecules that in turn may provide therapeutic agents for the treatment of certain cancers.

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Supporting Information Available: Table S1 and Figures S1−S3 described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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